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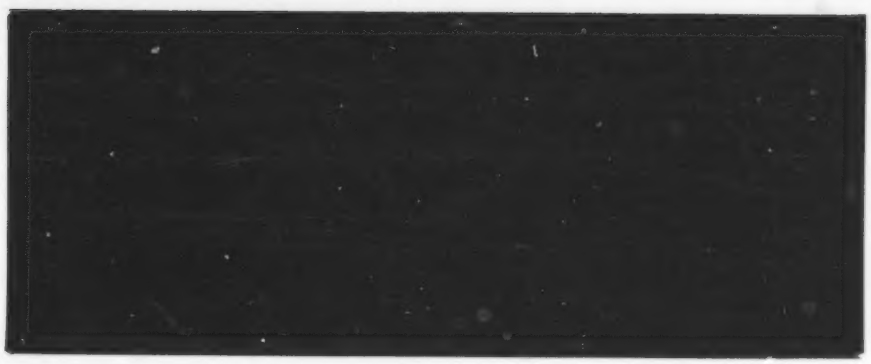

SASKATCHEWAN ALFALFA SEED PRODUCERS ASSOCIATION

TITLE : The Control of Chalkbrood and Other Fungal Problems of Leafcutter Bees

PROJECT NO.: 2.3-4

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Abstract

Saskatchewan leafcutter bee population surveys in 1986 and 1987 have shown that chalkbrood (*Ascosphaera aggregata*) is not present at detectable levels. However, a number of other fungal species have been isolated from *Megachile rotundata* adults, larval cadavers, nesting material, and nest provisions. Field experiments have demonstrated that moulds, yeasts and bacteria may be controlled by dipping nesting material and cocoons in chlorine bleach solution; nesting material may also be sterilized using kiln or microwave heating. Several fumigants tested have been found unsuitable for foliar mould control. Laboratory research has focused on determination of these moulds and yeasts which may be pathogenic to the leafcutter bee.

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SASKATCHEWAN ALFALFA
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SASKATCHEWAN ALFALFA SEED PRODUCERS ASSOCIATION
CHALKBROOD/FOLIAR MOULD PROJECT
FINAL RESEARCH REPORT

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TABLE OF CONTENTS

Abstract

Introduction

Research Methods and Results
I. Chalkbrood Survey
II. Investigation of Other Fungi in Leafcutter Bees
III. Development of Testing Methodology
IV. Evaluation of Existing Fungal Control Methods
V. Evaluation of Novel Fungal Control Methods

Discussion and Conclusions

Appendices

ABSTRACT

Research in this project involving Saskatchewan leafcutter bee populations has shown that chalkbrood (Ascosphaera aggregata) is not present at detectable levels in the province. However, a number of fungal species have been isolated from Megachile rotundata adults, larval cadavers, nesting material, and nest provisions. Field experiments have demonstrated that moulds, yeasts and bacteria may be controlled by dipping nesting material and cocoons in bleach solution; nesting material may also be sterilized using kiln or macrowave heating. Several fumigants tested have been found unsuitable for foliar mould control. Laboratory research has focused on determination of those moulds and yeasts which may be pathogenic to the leafcutter bee.

INTRODUCTION

The alfalfa leafcutter bee, Megachile rotundata, is a dependable pollinator of alfalfa and has been used by alfalfa seed producers in Saskatchewan for over twenty years. There are presently over 7500 ha devoted to alfalfa seed production, which peaked at 1.1 million kg in 1984, and new grower interest remains strong as farmers in the province seek to diversify their operations in response to the economic downturn affecting more traditional crops. In addition to sales of alfalfa seed, producers also market leafcutter bee cocoons, since populations increase each year under good management, allowing for sales of 500 million cocoons worth $2.5 million annually.

The major market for Saskatchewan leafcutter bee cocoons has been the United States. Since 1972 when a fungal disease known as chalkbrood (Ascosphaera aggregata) was first discovered in American leafcutter bees, growers in some alfalfa seed-producing regions of the western United States have been forced to import leafcutter bee cocoons as bee losses exceeded 50% in some states. Canadian populations of alfalfa leafcutter bees remained free of chalkbrood until the disease was found in Manitoba in 1982 and in southern Alberta in 1983. Chalkbrood surveys conducted by Saskatchewan Agriculture during the priod 1982-1984 detected A. aggregata at low levels in one Saskatchewan leafcutter bee population in 1984.

An outbreak of chalkbrood disease would make maintenance of sufficient leafcutter bee populations for alfalfa pollination in Saskatchewan difficult and would jeopardize the reputation for high quality enjoyed by Saskatchewan bees on the world market.

In addition to the fungus A. aggregata, a number of other fungi commonly occur in leafcutter bee populations; the diversity and abundance of micro-organisms associated with M. rotundata are not well documented but it is known that the presence of some mould and yeast species at high levels may be pathogenic to the alfalfa leafcutter bee. In order to investigate chalkbrood and other fungi occuring in Saskatchewan alfalfa leafcutter bee populations, a research project was undertaken with the following objectives:

1. To continue and broaden the annual chalkbrood survey begun in 1982 by Saskatchewan Agriculture.
2. To identify and monitor other fungi present in Saskatchewan alfalfa leafcutter bee populations and to attempt to determine their effect on leafcutter bee viability.
3. To develop a valid procedure for use in determining efficacy of fungal control methods.
4. To examine the efficacy and economics of existing methods used for control of fungi in leafcutter bee nesting material.
5. To evaluate new methods of fungal control(i.e. macrowave treatment, microwave treatment, fumigation treatment) which are not presently in common use.

RESEARCH METHODS AND RESULTS

I. Chalkbrood Survey

In order to assess the health of Saskatchewan leafcutter bee populations, samples of leafcutter bee cocoons from the 1985 and 1986 field seasons were solicited from alfalfa seed producers throughout the province. Samples were stored at about 4°C upon acquisition; from each sample, four 15 g subsamples were arrayed between pairs of polystyrene nesting boards. Sets of nesting boards were then taped shut, labelled, and x-rayed with a Hewlett-Packard Faxatron Unit (exposure 14 kvp @ 10 sec) using Kodak X-Omat AR x-ray film (20.1 x 25.2 cm format). Analysis of subsamples involved evaluation of x-rays with a Picker FVS single x-ray illuminator. All cocoons not containing healthy M. rotundata prepupae were marked on the x-ray, dissected, and their contents analysed and tabulated. Samples of moulds occurring on larvae, prepupae, pollen, and foliar material were set aside for detailed study, isolation, and identification. Other sample material was autoclaved following completion of analyses. Survey categories are listed below in Table I. Data collected were tabulated and mean, standard deviation and range calculated for each category; these detailed data are presented in Appendix A.

TABLE 1. 1983-1986 CHALKBROOD SURVEY DATA SUMMARY (%)[a]

Category	1983	1984	1985	1986
Healthy Prepupae	77.1	80.0	76.1	71.8
Dead Larvae/Prepupae				
a) Discoloured	3.9	3.2	5.0	6.0
b) Mouldy	0.4	0.3	0.5	0.6
c) Ascosphaera aggregata	-	0.003	-	-
d) Other Ascosphaera spp.	0.065	0.047	0.021	-
Mouldy Cocoons				
a) Pollen chalkbrood	1.0	0.4	0.5	0.3
b) Pollen mould	3.9	2.1	1.9	5.0
Parasites	1.1	0.4	0.5	1.5
Second Generation	1.0	0.6	0.9	0.5
Pollen Balls	10.2	11.3	12.0	13.1
Crushed Cocoons	1.2	2.3	2.2	1.1
Other[b]	n/a	n/a	0.5	0.2
Live count per kg	8630	9046	8580	8277
No. of samples	38	51	60	42

[a] Percent values may not total 100 due to truncation
[b] Category includes Coelioxys spp. parasites and wild Megachile spp.

No classical chalkbrood (Ascosphaera aggregata) was detected in surveys of leafcutter bees from the 1985 and 1986 field seasons. Occurrence of Ascosphaera spp. on larval cadavers in 1985 was limited to A.larvis and A.variegata, two native species which are not apparently pathogenic to M.rotundata but rather saprophytic in nature, growing on dead or moribund larvae. These species were not observed on cadavers in the 1986 survey. Ascosphaera species associated with pollen included A.pollenicola, A.variegata, A.larvis, and A.atra. Research carried out in collaboration with the Biosystematics Research Institute in Ottawa on larval and pollen specimens has indicated that there may be several native Ascosphaera species associated with leafcutter bee populations, and that isolation of new species will continue.

A comparison of survey data from 1983 to 1986 reveals a decrease in percent healthy prepupae for 1986 which corresponds to rises in pollen mould, pollen ball and discoloured larvae levels. Pollen balls occur when nectar and pollen provisions are collected by the female leafcutter bee but no egg is subsequently laid, or an egg or early instar larva collapses. Wet and cool weather which decreases leafcutter bee activity may be implicated in the high rate of pollen balls observed. These nectar and pollen provisions often provide an ideal environment for the growth of moulds and yeasts. A number of fungi isolated from mouldy pollen and larval cadaver samples were identified and will be discussed in the next section of this report.

The incidence of dead discoloured larvae (6% in 1986) is significant but poorly understood; this mortality may be related to environmental causes (i.e. temperature extremes) which effect the metabolism of the developing larvae or the condition of the provisions on which the larvae feed. The possibility that micro-organisms could play a role in this condition, which manifests itself as a hardened larval cadaver of beige, amber, or black colour with no extant mycelial growth, is presently under investigation. The remaining survey categories, including parasites (primarily the chalcid wasp, Pteromalus venustus), second generation (i.e. bivoltinism, the tendency for a small part of the population to emerge from cocoons prior to an overwintering period), and crushed cocoons (mechanical damage during harvest of cocoons) are problems which occur at low levels annually but do not significantly affect the quality of alfalfa leafcutter bee populations in Saskatchewan.

The 1987 chalkbrood survey will be carried out during the period November,1987 to March, 1988 as samples of leafcutter bee cocoons are received from producers throughout the province. Cool temperatures during the summer of 1987 have caused a noticeable decrease in bee activity, as in 1986, and the effect of these conditions on incidence of pollen and larval moulds will be studied carefully.

II. Investigation of Other Fungi in Leafcutter Bees

Moulds, yeasts, and bacteria associated with leafcutter bee populations in Saskatchewan were cultured, isolated, and identified according to a system based on source of inoculum. This classification method, based on various components of the leafcutter bee system (Table II), was necessary in order to separate fungal species and compare their occurrence from diverse sources.

TABLE II. LEAFCUTTER BEE MYCOFLORA CODING SYSTEM

Classification Code	Source of Inoculum
NM	Polystyrene or wood nesting material
CA	Larval cadavers, pollen specimens
AD	Adult males, females
CE	Cocoon exteriors, foliar material

Sampling of nesting material (NM series) was initially carried out in conjunction with experiments involving mould control in polystyrene and wood laminate nesting material. Samples taken in the field were cultured in the laboratory on various media, including PDA (Potato Dextrose Agar), SDA (Sabouraud's Dextrose Agar), and NA (Nutrient Agar). Colony types were isolated via sterile loop transfer, recultured, and then subcultured as purified isolates on PDA slants for storage at about 4°C. Mycoflora (i.e. moulds, yeasts, and bacteria) were isolated from many types of nesting material, including various types of polystyrene and wood solid blocks, during the course of the project. Mycoflora isolated from nesting material are listed in Appendix B.

Work on the CA series has involved extensive sampling of micro-organisms occuring on cadavers (larval,prepupal) and pollen samples. Most of this material has been collected from chalkbrood survey samples. Cultures are made by sterile loop transfer directly from the specimen to the culture plate. Purification of isolates is carried out as described above. A number of specimens, including those exhibiting larval, pollen, and foliar moulds,were photographed on daylight Ektachrome EM100 slide film using a Zeiss phototube and Pentax LX camera back with fibre optic and synchronized flash lighting. Representative specimens were then sent to Ottawa for detailed identification of fungi present. Identifications of CA series fungal isolates are catalogued in Appendix C.

In order to assess mycoflora associated with adult leafcutter bees (AD series), experiments were done involving trapping of working females in alfalfa fields and sampling of emergent males and females in the laboratory. In field studies, females were captured while entering, exiting, or capping

tunnels in nest boxes and were placed in 1 dram glass vials containing 1.0 ml sterile distilled H_2O. Following 30 sec of hand-agitation, bees were released and samples transferred to the laboratory, where 0.1 ml aliquots were plated on PDA and incubated ca 36h. In laboratory experiments with newly emerged male and female leafcutter bees, individuals were placed in test tubes containing 1.0 ml sterile dH_2O, vortexed ca 30 sec, and removed from the sample; again, 0.1 ml aliquots were cultured for 36h on PDA. Fungi and bacteria isolated from these samples (AD series) are recorded in Appendix D.

The final component of the leafcutter bee system to be analyzed was the CE series (i.e. mycoflora occurring on foliar exterior of cocoons). Samples of cocoons were selected from those received for chalkbrood surveys. From each sample a 50 ml subsample (ca 140 cocoons) was placed in a 30 dram crystallite vial to which 25 ml sterile distilled H_2O was added. Following a 30 sec vortexing, 0.1 ml aliquots were plated on PDA and incubated for 48h at ca 30°C. Micro-organisms isolated from leafcutter bee cocoons originating at various locations throughout the province are listed in Appendix E.

Species isolated from all four series described above were examined in lactophenol cotton blue mounts and preliminary identifications made using dichotomous keys; bacteria were gram stained. As previously mentioned, purified isolates were transferred to PDA slants for storage. Duplicate slants of each isolate were sent to the Biosystematics Research Institute, Ottawa for precise identification. A pure culture of each isolate was photographed on Ektachrome 100 slide film using a Pentax Spotmatic camera equipped with a wide angle lens and filtered for tungsten flood-lighting.

A variety of moulds, yeasts, and bacteria were isolated from different components of the leafcutter bee system. The most commonly occurring moulds include Aspergillus niger, Alternaria alternata, Eurotium chevalier, Rhizopus oryzae, and several Penicillium spp.; these are all generally saprophytic on provisions or on bees killed by other causes. Aspergillus and Rhizopus spp. have been reported to cause considerable damage in wild bee populations by spoiling provisions, while Trichothecium roseum and several Aspergillus spp. are known to act as facultative parasites of bees. Ascosphaera spp., discussed previously in the chalkbrood survey section, were isolated primarily from larval cadavers and pollen balls; the species identified are considered primarily saprophytic in nature, and often occurred in association with other fungi. Trichosporonoides was by far the most common yeast, occurring in every component of the leafcutter bee system. This genus of black yeasts is poorly known and requires taxonomic revision. Although the strains isolated were quite variable in cultural characteristics and morphology, they probably represent a single species which has yet to be described. A Saccharomyces sp. yeast was also cultured from nesting material. Several bacteria, including Erwinia, Pseudomonas, and Bacillus spp. were found in nesting material, on adults, and on cocoon exteriors.

Trap-nests were placed at locations throughout the central and southern parts of the province in early summer, 1986 in order to monitor the occurrence of micro-organisms in alfalfa seed-producing areas of Saskatchewan.

At each of twelve sites, nests were deployed in leafcutter bee shelters and nearby bluffs, left in place through the field season, and retrieved in the fall. Cocoons stripped from the trap-nests were analysed for viability and mycoflora present were isolated. No chalkbrood cadavers were detected in domestic or wild leafcutter bee cocoons sampled and mould levels in wild bees (e.g. M.relativa) were lower than levels found in M.rotundata, indicating that wild leafcutter bees are not likely carrying fungi into domestic populations. Micro-organisms isolated from nesting material varied among locations (Table III) with Aspergillus, Rhizopus, and Trichosporonoides spp. occurring most abundantly and bacterial growth heaviest in southern Saskatchewan.

TABLE III. MYCOFLORA ASSOCIATED WITH M.rotundata - OCCUPIED TUNNELS

Location	Predominant Mycoflora[a]
Cutknife	Erwinia sp., Rhizopus sp.
Parkside	Trichosporonoides sp., Aspergillus sp., Alternaria sp.
Spruce Home	- [b]
Macdowall	Trichosporonoides sp., Aspergillus sp.
Tisdale	Erwinia sp., Penicillium sp., Aspergillus sp.
Zenon Park	Misc. bacteria (spp.indet.)
Watson	Trichosporonoides sp., Erwinia sp.
Atwater	Trichosporonoides sp., Erwinia sp., Aspergillus sp.
Balcarres	Erwinia sp., misc. bacterium (sp.indet.)
Moose Jaw	Trichosporonoides sp., Erwinia sp.
Simmie	Trichosporonoides sp., Erwinia sp., Penicillium sp., Rhizopus sp.
Val Marie	Trichosporonoides sp., Penicillium sp.

[a] Micro-organisms listed in diminishing order of occurrence
[b] Only M.relativa present in trap-nests at this location

Analysis of data collected through deployment of trap-nests is useful in gaining a perspective on variation in leafcutter bee-related mycoflora between widely separated alfalfa seed-producing locations in the province. Trap-nests were again placed at a number of locations in the 1987 field season (ref. to map, Appendix F) and the contents of this new set of trap-nests will be analyzed later this year.

Experiments were initiated to evaluate the mycoflora occurring in fresh cell provisions, in fecal material deposited by larvae prior to cocoon-spinning, and on adult bees newly-emerged from their cocoons, to determine the effect on leafcutter bee viability of some moulds, yeasts and bacteria isolated from the system. While cultures of samples taken from these sources yielded numbers and species of micro-organisms which varied widely among individuals, the most commonly isolated included Aspergillus niger, Eurotium chevalieri, Trichosporonoides sp., Erwinia sp., and several other yeasts and bacteria. To assess the pathogenicity of A.niger spores towards M.rotundata prepupae (i.e. larvae which have spun their cocoons and are overwintering prior to pupation), a bioassay experiment was undertaken whereby a number of healthy individuals were dissected from their cocoons, placed in wells of sterile tissue culture plates and treated with 1 ul

droplets of distilled H_2O(control) or distilled H_2O containing 1.0×10^3 or 1.0×10^5 viable A.niger spores. Spores used in the bioassay were harvested from a PDA culture isolated from a mouldy larval cadaver. Delivery of 1ul droplets was accomplished with a 4 ul Drummond micropettor. Droplets were placed on the lateral portion of the prepupal integument and allowed to dry, whereupon the culture plate was placed in a 30°C incubator. The prepupae were assessed daily and by day 10, pupation had occurred in >50% of individuals in each treatment. In A.niger-treated pupating individuals the dark spores were visible in sloughed-off prepupal cuticle adhering to the tip of the abdomen. Development to mature adults was completed by day 24 in all treatments. Mortality of 5.0% in each of the control and 1×10^3 spore treatments was attributed to pre-treatment handling which caused haemorrhaging and subsequent prepupal deterioration. Under the conditions of the experiment, A.niger was shown to be non-pathogenic when applied topically to developing prepupae. Spores of A.niger were apparently unable to germinate upon or penetrate the protective cuticle of prepupal M.rotundata.

A second bioassay was carried out to determine the effect of peroral treatment with A.niger spores, Trichosporonoides sp. cells, and Saccharomyces sp. cells upon development of first instar larvae of the leafcutter bee. In this experiment, cells containing viable eggs and first instar larvae were extracted from a trap-nest in an alfalfa seed field near Saskatoon. Leaf caps were removed from the cells and 100 individuals were held at ca 25°C for 24h in order to assess their age and health. Individuals were then placed in sterile tissue culture plates and treated perorally with fungal spore or yeast cell preparations. Twenty-five remained as untreated controls, while other groups of twenty-five received 0.615×10^5 viable A.niger spores, 0.50×10^5 Trichosporonoides cells, or 0.50×10^5 Saccharomyces cells per individual. All treatment doses were delivered as 1ul volumes pipetted onto the surface of the semi-liquid provisions in close proximity to the M.rotundata egg or first instar larva. Tissue culture plates containing the developing larvae were held at room temperature (ca 25°C). Individuals were followed through a series of developmental stages including egg, pre-larval, early instar larval, mid instar larval, late instar larval-defecating, late instar larval-spinning, and spinning-completed phases. Differences in mean development time between untreated control larvae and those in the three treatments were not significant (Table IV), indicating that consumption of high levels of these mould spores and yeast cells was not apparently detrimental to the health of developing larvae. Observations of developing larvae yielded little pathological data; although lesions or yeast-like growths were observed on the cuticle of three larvae in the Saccharomyces sp. treatment, this did not prevent them from spinning cocoons. Individuals from all treatments are presently in prepupal diapause, with half stored at 30°C and half at 7°C. Incubation experiments will be conducted with these prepupae to assess possible mortality in prepupal or developing pupal stages which may be related to larval consumption of mould spores or yeast cells.

TABLE IV. TIME REQUIRED TO REACH COMPLETION OF COCOON SPINNING

Treatment	n[a]	Mean time(days)	Range (days)
Control	23	9.6	8-11
Aspergillus niger	22	10.0	8-14
Trichosporonoides sp.	23	8.9	6-11
Saccharomyces sp.	24	10.2	8-12

[a] individuals collapsing prior to feeding were deleted from the bioassay

III. Development of Testing Methodology

A method was developed for sampling and culturing micro-organisms present in leafcutter bee nesting material prior to and following fungal control treatments. Sterile cotton-tipped wood applicators (Diamond Peerless) 15 cm in length were dipped in sterile distilled H_2O and used to swab nest tunnels. The top 5 cm of each applicator was then removed to avoid contamination due to handling and the remainder of the applicator was placed in a sterile, screw-top, glass culture tube (Kimax,150 mm) which was then stored in a cooled container for transport to the laboratory. Applicators within tubes were reconstituted with 2.0 ml sterile distilled H_2O, vortexed ca 30 sec and plated in 0.1 ml aliquots on culture media. Three media (PDA, SDA and NA) were tested and PDA was found to be the best general purpose media for growth of moulds, yeasts, and bacteria. Cultures were usually incubated at 28 to 30°C for ca 48h. This sampling method was found to be extremely sensitive and was used in fungal control experiments involving several types of polystyrene laminate, wood laminate, polystyrene solid block, and wood solid block nesting material.

Two methods were developed for sampling micro-organisms on the surface of leafcutter bee cocoons. The first involved use of sterile cotton-tipped wood applicators which were moistened with sterile distilled H_2O and used to swab individual cocoons or cocoon series, particularly from samples showing heavy growth of foliar mould. The cotton tips were then applied directly to PDA media, which were incubated at ca 30°C for 24 to 48h. This method of isolating fungi and bacteria is not quantitative, but is a rapid technique for establishing identity of species present. It is useful for screening samples coming into the laboratory. A second, more precise method involved placing cocoons in sterile vials or culture tubes, adding known quantities of sterile distilled H_2O, vortexing, and then decanting the liquid for plating in 0.1 ml aliquots on PDA. Cultures were incubated ca 48h at 28 to 30°C. Both of the above methods were useful in evaluating foliar mould control achieved in cocoon treatment experiments.

IV. Evaluation of Existing Fungal Control Methods

At the present time in Saskatchewan, foliar mould control in nesting material is accomplished by dipping material in a sodium hypochlorite bleach solution using various tanks designed for this purpose. Probably fewer than half of the alfalfa seed producers in the province utilize this treatment method for several reasons. Bleach dipping is most compatible with those operations using polystyrene nesting material, since wood material will tend to warp and check if not properly dried following wetting. As well, sodium hypochlorite bleach is a caustic substance which can be difficult to work with, and the dipping process is time-consuming. The cost of dipping nesting material, which averages about 25 cents per nest box,was not considered by producers to be prohibitive. A new design of diptank, utilizing a rotating wheel to carry nest boxes through a semi-circular tank containing bleach solution, has recently been introduced in Saskatchewan. This design is portable, minimizes labour, and conserves dipping solution. It may well increase interest among alfalfa seed producers and lead to more widespread dipping of nesting material for control of foliar moulds.

A study was undertaken to determine the efficacy of dipping polystyrene nesting material in sodium hypochlorite bleach solution for control of

fungi. Sampling was carried out on a farm near Parkside, Sask. in late spring as nest boxes were being dipped prior to field placement. A number of undipped boxes were selected at random and several tunnels were swabbed from each of these boxes, using the method described in the previous section. Following the dipping operation, in which boxes moved through a tank containing ca 4% active chlorine and were inundated for about 3 min, another group of nest boxes was selected at random, allowed to dry, and sampled in the same fashion. Samples were transferred to the laboratory and cultured on PDA at 30°C for ca 36h. Analysis of cultures indicated that Aspergillus niger, Trichosporonoides sp., and bacteria including Pseudomonas sp. had been virtually eliminated from bleach-treated nesting material. These results clearly established the efficacy of sodium hypochlorite bleach treatment for control of moulds, yeasts, and bacteria in polystyrene nesting material.

A small number of producers in the province also dip their leafcutter bee cocoons in bleach prior to incubating them in the spring. A series of experiments was carried out to assess the efficacy of this method in controlling moulds present on cocoon surfaces and to determine whether bleach dipping had a deleterious effect on prepupae within the cocoons. Samples of cocoons known to contain healthy diapausing prepupae were left undipped (nontreated control), dipped in distilled H_2O (treated control) or dipped in one of a series of bleach solutions (1.0%, 2.5%, or 5.0% active chlorine). Following an inundation period of 3 min, cocoons were drained and air-dried. Subsequent sampling for foliar moulds indicated that all bleach treatments were highly effective in eliminating mould and yeast spores from cocoon surfaces. Control samples were heavily contaminated with Aspergillus niger and Trichosporonoides sp., although total colony numbers were much higher in the nontreated than in the H_2O-dipped cocoons, indicating that there is a substantial cleansing effect (i.e. removal of foliar contaminants) in the washing of cocoons. Air-dried cocoons were placed in an incubation chamber (ca 28°C) and percent emergence was determined to be 95 to 100% in all treatments. Clearly, the viability of dispausing prepupae was unaffected by sodium hypochlorite bleach treatment.

Further experiments carried out with dipped and undipped cocoons obtained from producers indicated that even in large-scale dipping operations, with the possibility of re-contamination during air-drying, levels of micro-organisms on dipped cocoons were $< 1.0\%$ of those found on nontreated samples. This reduction in foliar contamination had a great impact on the number of contaminating spores found on adult leafcutter bees, particularly females, emerging from incubation trays. Data showed that total spore number on females emerging from trays of dipped cocoons amounted to 6% of that found on females emerging from trays of nondipped cocoons.

V. Evaluation of New Fungal Control Methods

In order to develop alternative methods to sodium hypochlorite bleach dipping for control of micro-organisms in leafcutter bee nesting material, a number of potential decontaminating techniques were evaluated. These have included macrowave treatment, kiln heating, and the use of a number of fumigants (i.e.,ethylene oxide, methyl bromide,aluminum phosphide, and anhydrous ammonia).

Experimentation to evaluate macrowave treatment of wood nesting material (pine laminates) was undertaken on a farm near Leroy, Sask. Nest boxes were placed between electrode plates connected to a Mann Russell Electornic Generator (Model 200). The subsequent electromagnetic disturbance of wood molecules caused heating to temperatures of 65 to 80°C within time periods of 15 to 20 min. Tunnels were sampled before and after treatment and samples cultured as previously described. Evaluation of cultures (Table V) shows that moulds and yeasts were effectively controlled at macrowave-induced temperatures of 70°C and higher whereas at 65°C (corresponding to a shorter treatment time period) organisms from all three groups survived.

TABLE V. EFFICACY OF MACROWAVE TREATMENT FOR CONTROL OF MYCOFLORA

Temp. (°C)	Survival of micro-organisms		
	Moulds	Yeasts	Bacteria
65	+	+	+
70	-	-	+
80	-	-	+

It should be noted that numbers of surviving bacteria at the two higher treatment temperatures were extremely low. Macrowave treatment can obviously produce sufficient heat to control moulds and yeasts. Problems with this method of treatment are the lack of availability of macrowave equipment and the common occurrence of voltage fluctuation in rural areas; this latter factor may have tripled the time required to reach effective temperatures.

Laboratory research on the efficacy of kiln heating as a method for controlling micro-organisms was begun by treating solid wood block nesting material at temperatures of 50,75,100 and 125°C using a Fisher Isotemp Oven (Model 126G). Sampling of tunnels following 24h treatment periods (Table VI) shows that total control of fungi and bacteria is achieved at 100°C, while a temperature of 75°C is sufficient to eliminate moulds and yeasts, but not all bacteria. Moulds susceptible to temperatures of 75°C or greater include Rhizopus oryzae, Trichoderma citrinoviride, Aspergillus niger, Eurotium chevalieri, and Penicillium sp..

Further research to determine critical temperature and time treatments required to control micro-organisms in nesting material utilized both solid wood block and polystyrene laminate material.

TABLE VI. EFFICACY OF KILN HEATING FOR CONTROL OF MYCOFLORA

Temp. (°C)	Survival of micro-organisms		
	Moulds	Yeasts	Bacteria
50	+	+	+
75	-	-	+
100	-	-	-
125	-	-	-

Tunnels were sampled prior to treatment and following 6 and 12h at 80°C. Cultures of plated samples were evaluated after 48h at ca 28°C. In solid wood blocks, 6 and 12h treatments effectively controlled a wide range of fungal and bacterial species. In polystyrene nesting material, large numbers of bacteria survived the 6h treatment; while these numbers were somewhat lower after 12h, a variety of bacterial species were observed. Data indicate that Erwinia sp. and at least one other bacterium can survive a 12h/80°C treatment in polystyrene, but not in wood. The number of bacterial species cultured from polystyrene following a 12h/80°C treatment indicates that the heat may be activating some bacterial species (e.g. Chromobacter sp.) which would not otherwise germinate on PDA at 28°C.

Fumigation of nesting material, using ethylene oxide (ETO), methyl bromide (CH_3Br) and aluminum phosphide (AlP), was carried out using both wood and polystyrene material; additional work with anhydrous ammonia was done on polystyrene material only.

Nesting material used in ethylene oxide (Anprolene®) fumigation experiments was placed in a small vacuum chamber and exposed to a concentration of 200g ETO per m^3 for a 24h period at room temperature (ca25°C). Sampling of polystyrene laminate nesting material before and after treatment indicated that no micro-organisms survived. The same conclusion was reached in the treatment of solid wood block material, indicating that this gas is extremely effective in controlling moulds, including Rhizopus sp., Penicillium sp. and Aspergillus sp., as well as several dominant yeasts and bacteria. In an attempt to scale up the treatment process, additional work with ethylene oxide was carried out using a large vacuum chamber at the Western Development Museum in Saskatoon, Sask. The chamber (ca23.8m^3) was used with the fumigant Med-Gas®, a formulation containing 10% ETO and 90% CO_2. In this test, polystyrene laminate material was treated for 2.5h at a rate of 25 to 50 g ETO per m^3. Analysis of samples taken before and after treatment revealed no control of foliar moulds (e.g. Rhizopus sp., Aspergillus sp., Alternaria sp., Penicillium sp.) although yeast species and some bacteria were eliminated. The low concentration of ethylene oxide and the relatively short treatment time are likely explanations for the lack of mould control in this experiment. The extreme toxicity of ethylene oxide, combined with the high concentrations required, may limit its practicality for this application.

Fumigation with methyl bromide (Meth-O-Gas®) was undertaken at a producer location near Shellbrook, Sask., using a sealed incubation chamber of approximately 38m^3. Solid wood blocks and polystyrene laminate material were placed in the chamber, which was heated to 30°C prior to introduction of 908g of methyl bromide (24g CH_3Br per m^3). The temperature was held

above 27°C for 48h, then allowed to fall to ambient (-15°C) for 96h. The chamber was then aired for 24h before post-treatment sampling. Analysis of samples taken prior to and following fumigation indicates no clear-cut control of fungi in either wood or polystyrene material. The major yeast present (Trichosporonoides sp.) was eliminated from wood, while only bacteria appear to have been controlled in polystyrene. A second methyl bromide test was done at a farm near Zenon Park using a sealed chamber of ca14.5m^3. Solid polystyrene blocks were placed in the chamber and tunnels sampled. The chamber was then heated to 25°C and 908g of methyl bromide (62.6g per m^3) were relased into it. After 7 days, the chamber was opened and post-treatment samples were taken. No significant control of moulds, yeasts or bacteria was obtained, although the concentration of methyl bromide was 2.5 times that of the previous experiment.

Work with the fumigant aluminum phosphide (Phostoxin®) was undertaken near Shellbrook, Sask., using the incubation chamber described above. Solid wood blocks and polystyrene laminates were placed in the chamber along with sufficient aluminum phosphide tablets to release 30g of hydrogen phosphide gas. Temperature within the chamber was held at 20°C for 72h, then allowed to cool for a further 72h before a 24h airing period prior to post-treatment sampling. Sample analysis revealed that while numbers of organisms appeared slightly lower following fumigation, no moulds, yeasts, or bacteria were totally eliminated.

Experimentation with anhydrous ammonia involved treatment of solid polystyrene blocks. A number of these blocks were placed in a specially-constructed wooden box into which ca 2.5kg of anhydrous ammonia was pumped. The box was then sealed and held at ca 20°C for 17h. Cultures of samples taken before and after treatment indicated that no micro-organisms were eliminated by exposure to anhydrous ammonia.

An overview of results obtained in the testing of sodium hypochlorite bleach, macrowave treatment, kiln heating, and fumigants for the control of mycoflora in various types of polystyrene and wood leafcutter bee nesting material is given in Appendix G.

DISCUSSION AND CONCLUSIONS

Extensive surveys of Saskatchewan leafcutter bee populations indicate that during the 1985 and 1986 field seasons, chalkbrood (Ascosphaera aggregata) was not present at detectable levels in the province. Over the same two years, levels of chalkbrood exceeding 14% were found in isolated southern Alberta populations of M.rotundata. The possibility of an accidental introduction of the disease is readily apparent; concern on the part of Saskatchewan alfalfa seed producers has created a demand for information on chalkbrood and other fungal diseases of leafcutter bees and for research on methods to control mycoflora present in leafcutter bee populations. A number of Ascosphaera species isolated from the leafcutter bee environment, including A.atra, A.larvis, A.pollenicola, and A.variegata appear to be native to Saskatchewan and saprophytic in nature, occurring primarily on pollen or on bee larvae in which mortality has been caused by other factors. Further investigation into the distribution and frequency of these species, which are only now being discovered and described, will lead to a better understanding of their relative importance to leafcutter bee populations. In the 1996 chalkbrood survey, these Ascosphaera species (sometimes referred to as pollen chalkbrood) were present in populations at a level of only 0.3%.

Fungi other than Ascosphaera species present a much greater problem to Saskatchewan alfalfa seed producers. Losses of leafcutter bee eggs and developing larvae to overgrowth of provisions by moulds and yeasts were as high as 26.9% in one 1986 sample (Appendix A). It has been reported in alkali bee research that yeasts may initiate fermentation of the provision, sometimes in association with bacterial spp. and Rhizopus sp. The egg or larva thereupon dies, and the cell contents are overgrown by secondary invaders such as Aspergillus sp., Eurotium sp., Penicillium sp., Ascosphaera sp., and Mucor sp. All of these species, and yeasts including Saccharomyces and Trichosporonoides spp., have been isolated from mouldy pollen specimens and larval cadavers (Appendix C). The concurrent isolation of many of these fungi from individual specimens may indicate that a similar pathogenesis occurs in the alfalfa leafcutter bee.

While a number of different moulds and yeasts were found in various components of the leafcutter bee system, species such as Aspergillus niger, Eurotium chevalieri, Rhizopus oryzae, and Trichosporonoides sp. were most commonly cultured, along with the bacterium Erwinia sp.. All of these species were found at various levels throughout the province in trap-nests deployed for the purpose of monitoring mycoflora at diverse alfalfa seed producing locations. Relative abundance of these micro-organisms probably fluctuates with changing temperature and moisture conditions. Bioassay experiments undertaken to determine the effect of topical treatment of Aspergillus niger spores showed that mould to be non-pathogenic to healthy M.rotundata prepupae. Bioassays in which developing M.rotundata larvae were treated perorally with A.niger spores, Trichosporonoides sp. cells, and Saccharomyces sp. cells did not demonstrate pathogenicity up to the prepupal stage.

All of the moulds, yeasts, and bacteria isolated from leafcutter bee nesting material were found to be susceptible to sodium hypochlorite bleach dipping. This treatment method was also shown to be highly efficacious for surface decontamination of leafcutter bee cocoons. Experiments demonstrated that under large-scale producer dipping conditions, a 99% reduction in micro-organisms could be achieved. This reduction in mycoflora resulted in adult female M.rotundata leaving incubation trays carrying a substantially lower spore load (i.e. 6% of that load on females emerging from trays of undipped cocoons). Producers using polystyrene nesting material should utilize bleach dipping on an annual basis in order to prevent a potentially damaging build-up of fungal spores, and all producers should incorporate dipping of leafcutter bee cocoons into their bee management regime. Research reported here shows that bleach treatment does not harm prepupae within cocoons, and field release of cleaner adult females will reduce levels of inoculum within the nesting material environment.

Among novel methods of foliar mould control tested, the efficacy of heat treatments at temperatures of 80°C or higher was clearly shown in wood nesting material. A large-scale kiln-heating or macrowave operation could provide a viable alternative to the producer not wanting to bleach dip wood laminate or solid wood block material. Several Saskatchewan producers have expressed interest in construction of specialized facilities for heat treatment of nesting material. Of the fumigants tested, only ethylene oxide provided control of moulds, yeasts, and bacteria. Although this fumigant is efficient as a decontaminating agent, it is also a potent carcinogen and its use is strictly regulated in Canada. The fumigants methyl bromide, aluminum phosphide, and anhydrous ammonia were all found to be inefficient in controlling foliar moulds. Alternative compounds which exhibit fungicidal and fungistatic properties, and may be more effective than fumigants, should be investigated in the future. Various volatile essential oils, including acyclic terpenoids and fatty acid derivatives, could prove beneficial for treating nesting material and cocoons of M. rotundata in order to enhance the survival of these valuable pollinators.

The importance of mould control in leafcutter bee operations extends beyond the obvious objective of cleaner bee populations. There is evidence that some of the fungi isolated from the leafcutter bee system are potentially hazardous to human health. Correlations between medically significant species and those found in M.rotundata samples were examined as isolates were identified. Aspergillus niger and Alternaria alternata have been implicated in bronchopulmonary diseases and allergic reactions, and along with Penicillium and Rhizopus species are among the most important fungal allergens. Health problems reported by some alfalfa seed producers in this province indicate that it is essential to take precautions against prolonged exposure to mycoflora associated with the alfalfa leafcutter bee. The application of appropriate control methods for the reduction of mould spore levels in nesting material and on cocoon surfaces will help to alleviate these health problems.

APPENDIX A.

1985 Chalkbrood Survey Data Summary (%)

Stat.	Healthy Cocoons	Dead Larvae/ Prepupae Discol.	Mouldy	CB[a]	Mouldy Cocoons Pollen	CB	Other	Parasites	2nd Gen:	Pollen Balls	Crushed Damaged	Other
$\bar{X}$	76.1	5.0	0.5	0.02	0.5	1.9	0.5	0.9	12.0	2.2	0.5
s.d.	12.2	3.6	0.8	0.08	1.0	2.7	1.2	1.3	7.9	3.3	0.8
high	92.1	21.1	3.9	0.40	3.8	12.6	6.5	6.8	45.5	16.4	5.2
low	33.2	0.8	ø	ø	ø	ø	ø	ø	4.0	ø	ø

1986 Chalkbrood Survey Data Summary (%)

Stat.	Healthy Cocoons	Dead Larvae/Prepupae Discol.	Mouldy	CB[a]	Mouldy Cocoons Pollen	CB	Other	Parasites	2nd Gen.	Pollen Balls	Crushed Damaged	Other
$\bar{X}$	71.8	6.0	0.6	ø	0.3	5.0	1.5	0.5	13.1	1.1	0.2
s.d.	10.9	2.8	0.6	ø	0.6	5.4	6.9	0.7	4.1	2.9	0.2
high	88.7	11.6	2.8	ø	2.4	26.9	44.9	2.9	21.5	18.0	0.8
Low	23.3	1.7	ø	ø	ø	ø	ø	ø	4.7	ø	ø

[a] CB refers to chalkbrood species other than Ascosphaera aggregata in these tables

APPENDIX B.

Identification of Nesting Material (NM) Series Isolates

Isolate Designation	Identification	Type of Micro-organism
NM-A/29.07.86	*Aspergillus niger* Van Tiegham	Mould
NM-B/29.07.86	*Pseudomonas* sp.	Bacterium
NM-C/29.07.86	*Trichosporonoides* sp.	Yeast
NM-D/29.07.86	*Trichosporonoides* sp.	Yeast
NM-E/29.07.86	Bacterium (sp.indet.)	Bacterium
NM-F/29.07.86	*Erwinia* sp.	Bacterium
NM-G/29.07.86	*Saccharomyces* sp.	Yeast
NM-H/29.07.86	*Penicillium spinulosum* Thom	Mould
NM-I/30.07.86	*Trichoderma citrinoviride* Bissett	Mould
NM-J/05.08.86	*Alternaria alternata* (Fr.)Keissler	Mould
NM-K/03.09.86	Imperfect yeast (sp.indet.)	Yeast
NM-L/23.10.86	*Bacillus* sp.	Bacterium
NM-M/24.10.86	*Rhizopus oryzae* Went.& Prinson Geerlings	Mould
NM-N/31.10.86	*Erwinia* sp.	Bacterium
NM-O/27.04.87	*Alternaria alternata*(Fr.)Keissler	Mould
NM-P/27.04.87	*Alternaria alternata*?(non-sporulating)	Mould
NM-Q/01.05.87	*Cylindrocarpon* sp. or related Hypocreales	Mould

APPENDIX C.

1. Identification of Larval Cadaver and Pollen(CA) Series Isolates

Isolate Designation	Identification	Type of Micro-organism
CA-A/15.08.86	*Eurotium chevalieri* Mangin	Mould
CA-B/15.08.86	*Trichosporonoides* sp.	Yeast
CA-C/15.08.86	*Aspergillus niger* Van Tiegham	Mould
CA-D/15.08.86	*Penicillium purpurogenum*	Mould
CA-E/15.08.86	*Trichosporonoides* sp.	Yeast

2. Identification of Fungi on Specimens submitted to Ottawa

Larval Cadavers	Pollen Samples
MOULDS	MOULDS
Aspergillus glaucus	*Aspergillus glaucus*
Aspergillus niger	*Aspergillus niger*
Eurotium chevalieri	*Eurotium chevalieri*
Penicillium simplicissimum	*Penicillium* sp.
	Rhizopus oryzae
Ascosphaera larvis	*Ulocladium* sp.
Ascosphaera variegata	
	Ascosphaera atra
	Ascosphaera larvis
	Ascosphaera pollenicola
	Ascosphaera variegata
YEASTS	YEASTS
Trichosporonoides sp.	*Saccharomyces* sp.
Yeast (sp.indet.)	*Trichosporonoides* sp.

APPENDIX D.

Identification of Adult (AD) Series Isolates

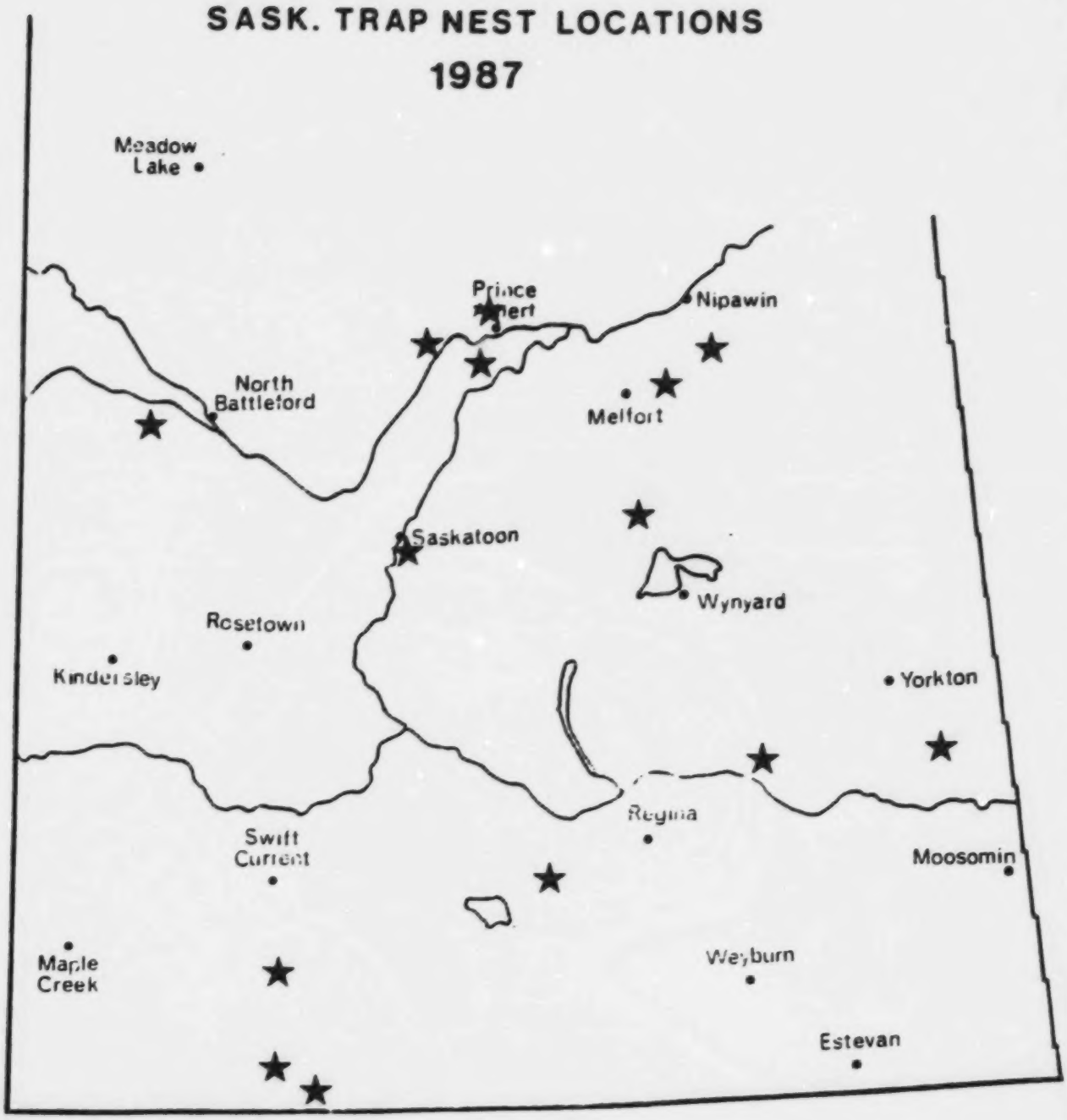
Isolate Designation	Identification	Type of Micro-organism
AD-A/05.09.86	*Rhizopus oryzae* Went.& Prinson Geerlings	Mould
AD-B/02.10.86	Yeast (sp.indet.)	Yeast
AD-C/02.10.86	*Trichosporonoides* sp.	Yeast
AD-D/02.10.86	*Erwinia* sp.	Bacterium
AD-E/02.10.86	*Alternaria alternata* (Fr.)Keissler	Mould
AD-F/02.10.86	Yeast	Yeast
AD-G/02.10.86	*Erwinia* sp.	Bacterium
AD-H/02.10.86	Bacterium (sp.indet.)	Bacterium
AD-I/02.10.86	*Alternaria alternata* (Fr.)Keissler	Mould
AD-J/02.10.86	*Aspergillus niger* Van Tiegham	Mould
AD-K/02.10.86	Bacterium (sp.indet.)	Bacterium
AD-L/25.04.87	*Eurotium* sp.(anam.Aspergillus glaucus group)	Mould
AD-M/25.04.87	*Eurotium chevalieri* Mangin	Mould
AD-N/25.04.87	*Ascosphaera* sp. (prob.variegata)	Mould
AD-O/25.04.87	*Trichothecium roseum* (Pers.)Link:Fr.	Mould

APPENDIX E.

Identification of Cell Exterior (CE) Series Isolates

Isolate Designation	Identification	Type of Micro-organism
CE-A/07.10.86	*Erwinia* sp.	Bacterium
CE-B/03.10.86	Bacterium (sp.indet.)	Bacterium
CE-C/03.10.86	*Trichosporonoides* sp.	Yeast
CE-D/03.10.86	*Mucor* sp.	Mould
CE-E/03.10.86	*Aspergillus niger* Van Tiegham	Mould
CE-F/03.10.86	*Penicillium* sp.	Mould
CE-G/07.10.86	*Erwinia* sp.	Bacterium
CE-H/03.10.86	*Alternaria alternata* (Fr.)Keissler	Mould
CE-I/03.10.86	Yeast (sp.indet.)	Yeast
CE-J/03.10.86	Yeast (sp.indet.)	Yeast
CE-K/03.10.86	Yeast (sp.indet.)	Yeast
CE-L/03.10.86	*Aspergillus niger* Van Tiegham	Mould

APPENDIX F.


SASK. TRAP NEST LOCATIONS
1987
Meadow Lake
Prince Albert
Nipawin
North Battleford
Melfort
Saskatoon
Wynyard
Rosetown
Kindersley
Yorkton
Regina
Swift Current
Moosomin
Maple Creek
Weyburn
Estevan

APPENDIX G.

Foliar Mould Control in LCB Nesting Material

Treatment Method	Nesting Material	Control
Bleach dipping	Polystyrene laminates	Control of moulds, yeasts, bacteria
Macrowave	Wood Laminates	Control of moulds, yeasts
Heating	Wood blocks	Control of moulds, yeasts, bacteria
	Poly.laminates	Control of moulds, yeasts
Methyl Bromide	Wood blocks	No substantial control
	Poly. laminates	No substantial control
	Poly. blocks	No substantial control
Ethylene Oxide	Wood blocks	Control of moulds, yeasts, bacteria
	Poly. laminates	Control of moulds, yeasts, bacteria
Med-Gas	Poly. laminates	Control of yeasts only
Aluminum Phosphide	Wood blocks	No substantial control
	Poly. laminates	No substantial control
Anhydrous Ammonia	Poly. blocks	No substantial control

The status of chalkbrood in Saskatchewan
and methods for control of fungal spores in alfalfa
leafcutter bee populations:

Practical information and recommendations for
alfalfa seed producers and extension personnel

Surveys conducted on Saskatchewan alfalfa leafcutter bee populations from the 1985 and 1986 field seasons indicate that the chalkbrood fungus, Ascosphaera aggregata, is not present at detectable levels in the province. However, high levels of chalkbrood in some southern Alberta leafcutter bee populations and continuing problems with the disease in alfalfa seed producing areas of the northwestern United States make the possibility of accidental introduction of chalkbrood disease into Saskatchewan a serious concern. The importation of leafcutter bee cocoons of nesting material into Saskatchewan from chalkbrood infested areas must be carefully guarded against.

At the present time, fungi other than chalkbrood constitute a much greater problem to alfalfa seed producers in the province than does chalkbrood. Losses of leafcutter bee eggs and developing larvae to overgrowth of nectar and pollen provisions by moulds and yeasts exceeded 25% in one 1986 sample. A number of these moulds and yeasts are common to all parts of Saskatchewan, and their relative abundance will fluctuate with changing temperature and moisture conditions.

Producers with polystyrene nesting material are advised to dip nest boxes in a 3 to 5% solution of sodium hypochlorite bleach for at least 3 minutes per box in order to decontaminate nest tunnels. Dipping of leafcutter bee cocoons in the same bleach solution will efficiently reduce surface contamination and result in cleaner bees emerging from incubation trays. Bleach treatment at concentrations up to 5% will not harm prepupae within cocoons. Producers using wood nesting material may also control foliar moulds by bleach dipping, or they may heat nesting material at temperatures above 80°C for a period of 24 hours. Other methods of decontaminating polystyrene and wood nesting material, including fumigation, have been shown to be unsatisfactory.

Control of foliar moulds in leafcutter bee operations is important for the health of both bee populations and producers. Many moulds found in bee samples can be hazardous to human health under conditions of prolonged exposure to high levels of spores. The application of control methods to nesting material will keep mould levels low and help to prevent these problems.

PUBLICATIONS

Newsletter Publications

Goerzen,D.W. 1986. Saskatchewan Chalkbrood Survey 1983-1985, p.4-5. SASPA Newsletter #86-2; June, 1986.

Goerzen,D.W. 1986. Mould Project Update/Efficacy of Dipping, p.7-8. SASPA Newsletter #86-3; August, 1986.

Goerzen,D.W. 1986. SASPA Chalkbrood and Foliar Mould Project Update, p.8-9. SASPA Newsletter #86-4; November, 1986.

Goerzen,D.W. 1987. Sask.Chalkbrood and Foliar Mould Research Project, p.22-27. SASPA Newsletter #87-1; February, 1987.

Goerzen,D.W. 1987. Survival of Bees after Bleach Treatment of Cocoons, p.9. SASPA Newsletter #87-2; June, 1987.

Proceedings/Extension Publications

Goerzen,D.W. 1987. Saskatchewan Chalkbrood and Foliar Mould Research Project, P.39-44. Proc.6th Canadian Alfalfa Seed School. 60p. Canadian Alfalfa Seed Council.

Murrell,D.C. and D.W.Goerzen. Chalkbrood and Foliar Mould Problems in Leafcutter Bees. Extension Bulletin.(manuscript in preparation).

Scientific Publications

Goerzen, D.W.and D.C.Murrell. The mycoflora of the alfalfa leafcutter bee, Megachile rotundata (Hymenoptera:Megachilidae) in Saskatchewan, Canada.(manuscript in preparation).

Goerzen,D.W. and D.C.Murrell. Pathogenicity of Aspergillus niger, Trichosporonoides sp., and Saccharomyces sp. to larvae of the alfalfa leafcutter bee, Megachile rotundata (Hymenoptera; Megachilidae). (manuscript in preparation).

Presentations

1) SASPA Annual Summer Field Day
   July 18, 1986 at Cutknife, Sask.
2) SASPA Annual Meeting
   Jan.4,5,1987 at Saskatoon, Sask.
3) 6th Canadian Alfalfa Seed School
   Jan.11-13,1987 at Edmonton,Alberta
4) SASPA Annual Summer Field Day
   July 31,1987 at Macdowall, Sask.

SASKATCHEWAN ALFALFA SEED PRODUCERS ASSOCIATION

Box 3003
Prince Albert, Sask.
S6V 6G1

Project 2.3-4: The Control of Chalkbrood and Other Fungal Problems of Leafcutter Bees

Funding Agency:

STATEMENT OF EXPENSES
April 1, 1987 - September 30, 1987

Expenditures

Fees - Wayne Goerzen		$14,000.00
Supplies and Equipment		61.71
Telephone		303.93
X-ray charges		ø
Miscellaneous		ø
	Total	$14,365.64

Total Expenditures April 1, 1986 - September 30, 1987

April 1 - September 12, 1986		$12,517.44
September 13 - March 31, 1987		17,247.42
April 1 - September 30, 1987		14,365.64
	Total	$44,130.50

Capital Equipment Purchased

One Light Table		$200.00
	Total	$200.00

Projected Expenditures October 1, 1987 - March 31, 1988

Fees - Wayne Goerzen		$14,000.00
Supplies and Equipment		200.00
Telephone		400.00
X-ray charges		1,000.00
	Total	$15,600.00

D Murrell

Dorothy Murrell
Secretary